

2020-04-06

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<http://hdl.handle.net/10026.1/16013>

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10.1016/j.niox.2020.03.007

Nitric Oxide: Biology and Chemistry

Elsevier

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# **Influence of muscle oxygenation and nitrate-rich beetroot juice supplementation on O<sub>2</sub> uptake kinetics and exercise tolerance**

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## Abstract

We tested the hypothesis that acute supplementation with nitrate ( $\text{NO}_3^-$ )-rich beetroot juice (BR) would improve quadriceps muscle oxygenation, pulmonary oxygen uptake ( $\dot{V}_{\text{O}_2}$ ) kinetics and exercise tolerance ( $T_{\text{lim}}$ ) in normoxia and that these improvements would be augmented in hypoxia and attenuated in hyperoxia. In a randomized, double-blind, cross-over study, ten healthy males completed two-step cycle tests to  $T_{\text{lim}}$  following acute consumption of 210 mL BR (18.6 mmol  $\text{NO}_3^-$ ) and  $\text{NO}_3^-$ -depleted beetroot juice placebo (PL; 0.12 mmol  $\text{NO}_3^-$ ). These tests were completed in normobaric normoxia [fraction of inspired oxygen content ( $\text{FIO}_2$ ): 21%], hypoxia ( $\text{FIO}_2$ : 15%) and hyperoxia ( $\text{FIO}_2$ : 40%). Pulmonary  $\dot{V}_{\text{O}_2}$  and quadriceps tissue oxygenation index (TOI), derived from multi-channel near-infrared spectroscopy, were measured during all trials. Plasma [nitrite] was higher in all BR compared to all PL trials ( $P < 0.05$ ). Quadriceps TOI was higher in normoxia compared to hypoxia ( $P < 0.05$ ) and higher in hyperoxia compared to hypoxia and normoxia ( $P < 0.05$ ).  $T_{\text{lim}}$  was improved after BR compared to PL ingestion in the hypoxic trials ( $250 \pm 44$  vs.  $231 \pm 41$  s;  $P = 0.006$ ;  $d = 1.13$ ), with the magnitude of improvement being negatively correlated with quadriceps TOI at  $T_{\text{lim}}$  ( $r = -0.78$ ;  $P < 0.05$ ).  $T_{\text{lim}}$  **was not** improved following BR ingestion in normoxia (BR:  $364 \pm 98$  vs. PL:  $344 \pm 78$  s;  $P = 0.087$ ,  $d = 0.61$ ) **or** hyperoxia (BR:  $492 \pm 212$  vs. PL:  $472 \pm 196$  s;  $P = 0.273$ ,  $d = 0.37$ ). BR ingestion increased peak  $\dot{V}_{\text{O}_2}$  in hypoxia ( $P < 0.05$ ), but not normoxia or hyperoxia ( $P > 0.05$ ). These findings indicate that BR supplementation is more likely to improve  $T_{\text{lim}}$  and peak  $\dot{V}_{\text{O}_2}$  in situations when skeletal muscle is more hypoxic.

**Key Words:** nitric oxide; vascular function; oxidative metabolism; exercise performance; fatigue; near-infrared spectroscopy

## 1. INTRODUCTION

Nitric oxide (NO) is a free radical that impacts a plethora of physiological processes in skeletal muscle [1,2] and has implications for skeletal muscle fatigue development [3,4]. Although the classical pathway for NO production is the oxidation of L-arginine by NO synthases (NOS) [5], dietary supplementation with L-arginine does not increase NO biomarkers and exercise performance in healthy humans [6-8] due to poor oral bioavailability of L-arginine [van de Poll et al., 2007 *Am J Clin Nutr*; Wu, 1998 *J Nutr*]. It is now recognised that NO can be derived from the stepwise and O<sub>2</sub>-independent reduction of inorganic nitrate (NO<sub>3</sub><sup>-</sup>) to nitrite (NO<sub>2</sub><sup>-</sup>) and subsequently to NO [9-10]. Acute or chronic dietary supplementation with NO<sub>3</sub><sup>-</sup> has been consistently demonstrated to elevate plasma [NO<sub>2</sub><sup>-</sup>], and can improve exercise economy, the kinetics of pulmonary oxygen uptake ( $\dot{V}_{O_2}$ ) following the onset of exercise, and/or exercise performance in healthy, moderately trained individuals in some [11-20], but not all [e.g., 15], studies in normoxia [see McMahon et al., 2017, *Sports Med* for a systematic review and meta analysis].

It is well documented that the one-electron reduction of NO<sub>2</sub><sup>-</sup> to NO is enhanced in conditions of acidosis [21] and hypoxia [22]. Since breathing a hypoxic inspirate has been reported to lower skeletal muscle conductive O<sub>2</sub> delivery [23], oxygenation [24] and pH [25] during exercise, greater NO<sub>2</sub><sup>-</sup> reduction to NO would be expected in this setting compared to normoxia. When exercising in normobaric hypoxia, dietary NO<sub>3</sub><sup>-</sup> supplementation has been reported to improve skeletal muscle oxygenation, exercise economy and exercise performance [24,26-28], and to improve matching between skeletal muscle O<sub>2</sub> supply and utilisation [29]. Moreover, exercise-induced perturbations to skeletal muscle metabolic homeostasis, such as the declines in phosphocreatine (PCr) and pH and increases in adenosine diphosphate (ADP) and inorganic phosphate (Pi), are restored towards normoxic responses after NO<sub>3</sub><sup>-</sup> supplementation in hypoxia [25]. Therefore, lowering skeletal muscle oxygenation might augment the potential for dietary NO<sub>3</sub><sup>-</sup> supplementation to improve physiological responses during exercise and endurance performance. However, in studies comparing the ergogenic effects of NO<sub>3</sub><sup>-</sup> supplementation in normoxia and hypoxia, it has been reported that NO<sub>3</sub><sup>-</sup> supplementation is ineffective at enhancing performance in normoxia and hypoxia [Bourdillon et al. 2015, *Front Physiol*; MacLeod et al., 2015, *Int J Sport Nutr Exerc Metab*; Nybäck et al., 2017, *Nitric Oxide*], equally effective at enhancing performance in normoxia and hypoxia [Rokkedal-Lausch et al., 2019, *Nitric Oxide*], and at more effective at enhancing performance

in hypoxia compared to normoxia [Kelly et al., 2014, Am J Physiol Regul Integr Comp Physiol]. Such inter-study discrepancies underscore the requirement of further research to address the relative ergogenic efficacy of  $\text{NO}_3^-$  supplementation in normoxia and hypoxia.

Breathing an  $\text{O}_2$  enriched gas mixture has been reported to lower plasma  $[\text{NO}_2^-]$  [30,31], which has been attributed to elevated oxidative stress and an associated increase in NO scavenging [31]. In addition, inhalation of hyperoxic gas has been reported to increase skeletal muscle conductive  $\text{O}_2$  delivery [23,32,33] and oxygenation [34], which would be expected to limit the reduction of  $\text{NO}_2^-$  to NO during exercise. Our group has recently reported that mouse single skeletal muscle fibres incubated with  $\text{NaNO}_2$  exhibit increased time to fatigue during evoked contractions at a physiological  $\text{P}_{\text{O}_2}$ , but lower time to fatigue at a supraphysiological  $\text{P}_{\text{O}_2}$  [35]. However, it is unclear whether dietary  $\text{NO}_3^-$  supplementation compromises exercise capacity when skeletal muscle oxygenation is increased in humans.

The purpose of the current study was to assess the effect of modulating skeletal muscle oxygenation on plasma  $[\text{NO}_2^-]$ , skeletal muscle oxygenation and pulmonary  $\text{O}_2$  uptake ( $\dot{V}_{\text{O}_2}$ ) kinetics during moderate-intensity exercise (completed below the gas exchanged threshold), and severe-intensity exercise (completed above the critical power) performed to the limit of tolerance ( $T_{\text{lim}}$ ), following acute dietary  $\text{NO}_3^-$  supplementation [36]. It was hypothesised that acute supplementation with  $\text{NO}_3^-$ -rich beetroot juice (BR) would increase muscle oxygenation and lower end-exercise  $\dot{V}_{\text{O}_2}$  during a moderate-intensity step exercise test in normoxia and that these effects would be augmented in hypoxia and abolished in hyperoxia. It was also hypothesised that BR supplementation would increase muscle oxygenation, expedite the adjustment in  $\dot{V}_{\text{O}_2}$  and improve  $T_{\text{lim}}$  during a subsequent step increment to severe-intensity exercise in normoxia and that these variables would be improved to a greater extent in hypoxia and to a lesser extent in hyperoxia.

## 2. METHODS

### 2.1 Subjects characteristics

Ten healthy non-smoking males (age,  $23 \pm 3$  yrs; body mass,  $78 \pm 9$  kg; height,  $1.80 \pm 0.07$  m; mean  $\pm$  SD) volunteered to participate in this study. The subjects participated in exercise at a recreational level but were not highly trained ( $\dot{V}_{\text{O}_{2\text{peak}}}$ ,  $49 \pm 5$  ml $\cdot$ kg $^{-1}\cdot$ min $^{-1}$ ). All subjects were

familiar with laboratory exercise testing procedures, having previously participated in studies employing cycle ergometry in our laboratory. The procedures employed in this study were approved by the University of Exeter Research Ethics Committee and all subjects were required to give their written informed consent prior to the commencement of the study after the experimental procedures, associated risks, and potential benefits of participation had been explained. Subjects were instructed to arrive at the laboratory in a rested and fully hydrated state, at least 3 h postprandial, and to avoid strenuous exercise in the 24 h preceding each testing session. All tests were performed at the same time of day ( $\pm 2$  h) and subjects were instructed to abstain from antibacterial mouthwash use for the duration of the study, as antibacterial mouthwash is known to attenuate  $\text{NO}_3^-$ -reductase activity by commensal bacteria in the oral cavity [37]. Subjects were also asked to avoid consumption of  $\text{NO}_3^-$ -rich (beetroot, celery, cress, lettuce, radish, rocket, spinach) and glucosinolate/thiocyanate-rich foods (*Brassica* vegetables such as broccoli, brussels sprouts cabbage and cauliflower) [38] for 48 h, and caffeine and alcohol ingestion for 12 and 24 h before each test, respectively.

## 2.2 Experimental Design

Subjects were required to report to the laboratory on nine occasions over a 4-7 week timeframe. After completing an initial ramp incremental exercise test and familiarization trials, subjects completed step cycling tests for determination of plasma  $[\text{NO}_2^-]$  and  $\dot{V}\text{O}_2$  kinetics, quadriceps oxygenation and exercise tolerance. These trials were completed after acute consumption of BR or placebo (PL) in normobaric normoxia (BR-Norm, PL-Norm), hypoxia (BR-Hypo, PL-Hypo) and hyperoxia (BR-Hyper, PL-Hyper), as described below in 2.5 *Supplementation Procedures* and 2.6 *Inspirate Generation*. These experimental conditions were administered in a quasi-double-blind (investigators were aware of the  $\text{FIO}_2$  but not the supplementation conditions), randomised, crossover experimental design.

## 2.3 Incremental Test

Before the intervention period, subjects completed a ramp incremental exercise test for determination of  $\dot{V}\text{O}_{2\text{peak}}$  and gas exchange threshold (GET). All exercise tests were performed on an electronically-braked cycle ergometer (Lode Excalibur Sport, Groningen, the Netherlands). Initially, subjects performed 3 min of baseline cycling at 20 W, after which the work rate was increased by  $40 \text{ W} \cdot \text{min}^{-1}$  until the limit of tolerance. The subjects cycled at a self-selected pedal rate (between 70-90 rpm) and this pedal rate along with saddle and

handlebar height and configuration was recorded and reproduced in subsequent tests. Breath-by-breath pulmonary gas-exchange data were collected continuously during the incremental tests and averaged over consecutive 10 s periods. The  $\dot{V}_{O_{2peak}}$  was taken as the highest 30 s mean value attained prior to the subject's  $T_{lim}$  in the test. The GET was determined from a cluster of measurements including: 1) the first disproportionate increase in  $\dot{V}_{CO_2}$  production ( $\dot{V}_{CO_2}$ ) from visual inspection of individual plots of  $\dot{V}_{CO_2}$  vs.  $\dot{V}_{O_2}$ ; 2) an increase in expired ventilation ( $\dot{V}_E$ ) /  $\dot{V}_{O_2}$  with no increase in  $\dot{V}_E$  /  $\dot{V}_{CO_2}$ ; and 3) an increase in end-tidal  $O_2$  tension with no fall in end-tidal  $CO_2$  tension. The data collected during the incremental test was used to calculate the work rates which were employed during the subsequent step exercise tests. Specifically, the work rates that would require 95% of the  $\dot{V}_{O_2}$  at GET (moderate-intensity exercise) and 75% of the difference between the  $\dot{V}_{O_2}$  at the GET and  $\dot{V}_{O_{2peak}}$  ( $75\%\Delta$ ); severe-intensity exercise) were estimated with account taken of the mean response time of the  $\dot{V}_{O_2}$  response to ramp exercise (i.e., two-thirds of the ramp rate was subtracted from the power output at the GET and  $\dot{V}_{O_{2peak}}$ ) [39].

## 2.4 Step Exercise Tests

Subjects completed a two-step exercise test in normoxia and hypoxia on visits two and three for familiarisation with the exercise protocol and all experimental procedures described below. On the remaining six laboratory visits, subjects completed a two-step exercise test in the BR-Norm, PL-Norm, BR-Hypo, PL-Hypo, BR-Hyper and PL-Hyper experimental conditions. Upon arrival at the laboratory, a cannula (Insyte-W Becton-Dickinson, Madrid, Spain) was inserted into a forearm vein to enable the collection of venous blood samples during and immediately after the two-step exercise test. Subjects then mounted the cycle ergometer and were fitted with a mouthpiece, fingertip pulse oximeter and three sets of near-infrared spectroscopy (NIRS) probes (see Measurements section below). Subsequently, subjects underwent 5 min of seated rest whilst inhaling one of the three experimental test inspirates (see Inspirate Generation section below) before commencing the two-step exercise test. Each two-step exercise test began with 2 min of baseline low-intensity 'unloaded' cycling at 20 W before an abrupt transition to a moderate-intensity constant work rate equivalent to 95% GET (U→M). Following 4 min of moderate-intensity cycling, the work rate was abruptly increased to a severe-intensity constant work rate equivalent to  $75\% \Delta$  (M→S). The severe-intensity exercise bout was continued to the limit of tolerance, which was recorded when the pedal rate fell more than 10 rpm below the required pedal rate.

## 2.5 Supplementation Procedures

Subjects were required to ingest 210 mL of a beetroot juice concentrate (Beet it, James White Drinks Ltd., Ipswich, UK) 2.5 hours prior to arrival at the laboratory for all experimental tests. For the BR-Norm, BR-Hypo and BR-Hyper trials, subjects ingested  $\text{NO}_3^-$ -rich beetroot juice concentrate (which provided  $\sim 18.6 \text{ mmol NO}_3^-$ ), whereas subjects ingested  $\text{NO}_3^-$ -depleted beetroot juice concentrate in the PL-Norm, PL-Hypo and PL-Hyper trials (which provided  $\sim 0.12 \text{ mmol NO}_3^-$ ). Each experimental test was separated by a washout period of at least 48 h [20].

## 2.6 Inspirate Generation

The hypoxic and hyperoxic inspirates administered in this study were generated using an air separation unit (CAT-12, Colorado Altitude Training, Louisville, USA). The system comprised two outlets, one which expelled  $\text{O}_2$  enriched air and one which expelled  $\text{O}_2$  depleted air. Air expelled from one of these outlets was delivered, via an extension conduit, to a 1000 L Douglas Bag (Cranlea & Co., Birmingham, UK), which acted as a reservoir and mixing chamber. For the hypoxic and hyperoxic trials, the  $\text{O}_2$  depleted and  $\text{O}_2$  enriched gases were mixed until the required  $\text{O}_2$  percentage was attained. For the normoxic trials, the air separation mode was turned off. The  $\text{O}_2$  and  $\text{CO}_2$  concentration of the inspire was monitored during each test using a Servomex 5200 High Accuracy Paramagnetic  $\text{O}_2$  and  $\text{CO}_2$  Analyzer (Servomex, Crowborough, UK). The gas analyzer was calibrated prior to each test with a 16.0%  $\text{O}_2$ , 8.0%  $\text{CO}_2$  and 76.0%  $\text{N}_2$  gas mix (BOC Special Gases, Guildford, UK). The  $\text{O}_2$  % in the PL-Norm, BR-Norm, PL-Hypo, BR-Hypo, PL-Hyper and BR-Hyper trials was  $21.0 \pm 0.2$ ,  $21.0 \pm 0.1$ ,  $14.9 \pm 0.1$ ,  $14.9 \pm 0.1$ ,  $40.1 \pm 0.1$  and  $40.0 \pm 0.1$ , respectively. The 1000 L Douglas Bag comprised a separate outlet tube that linked to a two-way breathing valve system (Hans Rudolph, Cranlea & Co.). The two-way valve was connected to the mouthpiece which provided a constant, unidirectional flow rate and ensured that no re-breathing of expired air occurred.

## 2.7 Measurements

Venous blood was sampled at the end of unloaded and moderate-intensity cycling, 120 s following the onset of severe-intensity cycling and at  $T_{\text{lim}}$  during severe-intensity cycling.



Samples were drawn into 6-ml lithium-heparin tubes (Vacutainer, Becton-Dickinson, New Jersey, USA) and centrifuged at 4000 rpm and 4°C for 10 min, within 2 min of collection. Plasma was subsequently extracted and immediately frozen at -80°C for later analysis of nitrite concentration ( $[\text{NO}_2^-]$ ) via ozone-based chemiluminescence as described previously [20].

Arterial  $\text{O}_2$  saturation ( $\text{SpO}_2$ ) was measured continuously at 0.5 Hz using a Rad-87 pulse oximeter (Masimo, Irvine, USA) attached to the right index finger and exported for later analysis. The spatially-resolved quadriceps tissue oxygenation index (TOI) was measured continuously at three sites using two continuous-wave NIRS systems with data sampled at 1 Hz. The TOI of the *rectus femoris* was assessed using a NIRO 200 tissue oxygenation spectrometer (Hamamatsu Photonics KK, Hamamatsu City, Japan) while the TOI of the *vastus lateralis* was assessed using a NIRO 200NX tissue oxygenation spectrometer (Hamamatsu Photonics KK, Hamamatsu City, Japan). Both systems comprised an emission probe that irradiated laser beams and a detection probe. Three different wavelength laser diodes provided the light source (775, 810 and 850 nm in the NIRO 200 and 735, 810 and 850 in the NIRO 200NX) and the light returning from the tissue was detected by a photomultiplier tube in the spectrometer. The optodes were placed in a holder, which was secured to the skin with adhesive. The *rectus femoris* probe was placed at 50% of the distance between the patella and the greater trochanter. The proximal *vastus lateralis* probe was attached at 70%, with the distal *vastus lateralis* probe attached at 30%, of the distance between the patella and the greater trochanter. To secure the holder and wires in place, an elastic bandage was wrapped around the subject's leg. The wrap helped to minimize the possibility that extraneous light could influence the signal and ensured that the optodes did not move during exercise. Indelible pen marks were made around the holder to enable precise reproduction of the placement in subsequent tests. The inter-optode distance (3 cm) and optical pathlength factor (18.6 cm) were consistent between measurement sites.

During all tests, pulmonary gas exchange and ventilation were measured breath-by-breath using an automated gas analysis system (CPX Express, MedGraphics, St. Paul, MN, USA). Subjects wore a nose clip and breathed through a low-dead-space, low-resistance mouthpiece and preVent pneumotach flowmeter assembly (MedGraphics, St. Paul, MN, USA). The inspired and expired gas volume and gas concentration signals were continuously sampled, the latter using galvanic ( $\text{O}_2$ ) and non-dispersive infrared ( $\text{CO}_2$ ) analyzers (CPX Express, MedGraphics, St. Paul, MN, USA) via a capillary line connected to the mouthpiece. The gas

analyzers were calibrated before each test with gases of known concentration and the turbine volume transducer was calibrated with a 3-liter syringe (Hans Rudolph, Kansas City, MO).

## 2.8 Data Analysis

The breath-by-breath  $\dot{V}_{O_2}$  data from each test were initially examined to exclude errant breaths caused by coughing, swallowing, sighing, etc., and those values lying more than four standard deviations from the local mean were removed. The breath-by-breath data were subsequently linearly interpolated to provide second-by-second values and time-aligned to the start of exercise. A single-exponential model without time delay, with the fitting window commencing at  $t = 0$  s (equivalent to the mean response time, MRT) was used to characterise the kinetics of the overall  $\dot{V}_{O_2}$  response during the U→M and M→S step work rate increments as described in the following equation:

$$\dot{V}_{O_2}(t) = \dot{V}_{O_2 \text{ baseline}} + A(1 - e^{-(t/MRT)}) \quad (\text{Eqn. 1})$$

where  $\dot{V}_{O_2}(t)$  represents the absolute  $\dot{V}_{O_2}$  at a given time  $t$ ;  $\dot{V}_{O_2 \text{ baseline}}$  represents the mean  $\dot{V}_{O_2}$  measured over the final 60-s of baseline; and  $A$  and  $MRT$  represent the amplitude and MRT, respectively, describing the overall increase in  $\dot{V}_{O_2}$  above baseline. An iterative process was used to minimise the sum of the squared errors between the fitted function and the observed values. We quantified the  $\dot{V}_{O_2}$  MRT with the fitting window constrained to the end of the U→M work rate increment and to 180 s of the M→S work rate increment. The absolute  $\dot{V}_{O_2}$  at the end (mean over the final 60s) of the U→M and M→S work rate increments, and at 180 s ( $\pm 15$  s) of M→S were also calculated, as was the change ( $\Delta$ ) in  $\dot{V}_{O_2}$  between baseline and end-exercise in the U→M and M→S work rate increments and between baseline and 180 s of the M→S work rate increment.

The TOI responses at the medial *rectus femoris* and proximal and distal *vastus lateralis* were averaged prior to analysis. The absolute TOI at the end (mean over the final 60s) of the unloaded baseline, the U→M and M→S work rate increments, and at 180 s ( $\pm 15$  s) during the M→S work rate increment were subsequently calculated. The  $SpO_2$  data from the start of unloaded cycling up to 120 s of severe-intensity cycling exercise were averaged for each experimental condition to provide an overall  $SpO_2$  profile for each trial.

## 2.9 Statistical Analysis

A two-way, supplement (PL and BR)  $\times$  inspire (hypoxia, normoxia and hyperoxia), repeated-measures ANOVA was employed to assess differences in plasma  $[\text{NO}_2^-]$ ,  $\dot{V}_{\text{O}_2}$  kinetics,  $\text{SpO}_2$ , TOI and exercise tolerance across the experimental conditions. Significant effects were further explored using post-hoc Fisher's LSD  $t$ -tests. Relationships between the outcome variables were assessed using Pearson's correlation coefficient ( $r$ ). Effect size for  $T_{\text{lim}}$  comparisons were assessed using Cohen's  $d$ . Data are presented as mean  $\pm$  SD, unless otherwise stated. Statistical significance was accepted when  $P < 0.05$ .

### 3.0 RESULTS

The BR and PL supplements and hypoxic and hyperoxic inspirates administered in this study were well tolerated with no side-effects reported. All participants self-reported that their dietary and exercise habits were consistent across the duration of the study. Participants attained a peak work rate of  $379 \pm 40$  W and  $\dot{V}_{\text{O}_2}$  of  $3.82 \pm 0.50$  L $\cdot$ min $^{-1}$  ( $49 \pm 5$  ml $\cdot$ kg $^{-1}\cdot$ min $^{-1}$ ) during the ramp incremental test. The work rates applied during the moderate-intensity and severe-intensity step cycle tests were  $106 \pm 14$  W and  $292 \pm 34$  W, respectively.

#### 3.1 Plasma $[\text{NO}_2^-]$

Plasma  $[\text{NO}_2^-]$  data are presented in figure 1. Plasma  $[\text{NO}_2^-]$  was higher at the end of baseline cycling, moderate-intensity cycling, 120 s of severe-intensity cycling and at  $T_{\text{lim}}$  in the BR-Hypo, BR-Norm and BR-Hyper trials compared to the PL-Hypo, PL-Norm and PL-Hyper trials ( $P < 0.01$ ); however, there were no differences between the BR-Hypo, BR-Norm and BR-Hyper trials or between the PL-Hypo, PL-Norm and PL-Hyper trials at specific time points ( $P > 0.05$ ). In the BR, but not PL trials, plasma  $[\text{NO}_2^-]$  was lower at  $T_{\text{lim}}$  compared to the end of baseline cycling, moderate-intensity cycling and 120 s of severe-intensity cycling ( $P < 0.05$ ).

#### 3.2 Arterial oxygen saturation

The mean  $\text{SpO}_2$  data across trials are presented in figure 2. Mean  $\text{SpO}_2$  was lower in the hypoxic trials compared to the normoxic and hyperoxic trials ( $P < 0.01$ ), but not different between the normoxic and hyperoxic trials ( $P > 0.05$ ).

#### 3.3 Tissue oxygenation index

Quadriceps TOI was higher during moderate-intensity and severe-intensity cycling exercise in the PL-Norm trial compared to the PL-Hypo trial ( $P < 0.05$ ) and in the PL-Hyper trial compared

to the PL-Norm and PL-Hypo trials ( $P<0.05$ ; Figure 3). There were no differences in quadriceps TOI between the PL-Hypo and BR-Hypo trials or between the PL-Hyper and BR-Hyper trials ( $P>0.05$ ), but quadriceps TOI was higher during baseline ( $71 \pm 3$  vs.  $69 \pm 3$  %) and moderate-intensity ( $67 \pm 5$  vs.  $65 \pm 4$  %) cycling exercise in the BR-Norm trial compared to the PL-Norm trial ( $P<0.05$ ; figure 4).

### 3.4 Pulmonary oxygen uptake

The group mean pulmonary  $\dot{V}_{O_2}$  data are presented in table 1 with the  $\dot{V}_{O_2}$  responses from a representative individual and the group mean end-exercise  $\dot{V}_{O_2}$  shown in figure 5. There were no differences in the absolute  $\dot{V}_{O_2}$  during baseline, moderate-intensity cycling or the first 180 s of severe-intensity cycling between any of the experimental conditions ( $P>0.05$ ). The  $\dot{V}_{O_2}$  MRT was longer following the onset of severe-intensity cycling initiated from a moderate-intensity baseline compared to moderate-intensity cycling initiated from an unloaded baseline ( $P<0.01$ ; table 1). However, the  $\dot{V}_{O_2}$  MRT during moderate-intensity and severe-intensity exercise were not different between experimental conditions ( $P>0.05$ ). The change in  $\dot{V}_{O_2}$  over the first 180 s of severe-intensity cycling and end-exercise  $\dot{V}_{O_2}$  during severe-intensity cycling were not different between the PL-Norm and PL-Hyper conditions ( $P>0.05$ ) but were lower in the PL-Hypo trial compared to both the PL-Norm and PL-Hyper trials ( $P<0.05$ ; table 1). There were no differences in the change in  $\dot{V}_{O_2}$  over the first 180 s or end-exercise  $\dot{V}_{O_2}$  during severe-intensity cycling between the BR and PL conditions in normoxia and hyperoxia ( $P>0.05$ ), but these variables were both higher after BR supplementation in hypoxia compared to PL supplementation in hypoxia ( $P<0.05$ ; table 1; figure 5).

### 3.5 Exercise tolerance

$T_{lim}$  was shorter in the normoxic trials (PL-Norm:  $344 \pm 78$  s, BR-Norm:  $364 \pm 98$  s) compared to the hyperoxic trials (PL-Hyper:  $472 \pm 196$  s, BR-Hyper:  $492 \pm 212$  s) and shorter in the hypoxic trials (PL-Hypo:  $231 \pm 41$  s, BR-Hypo:  $250 \pm 44$  s) compared to the normoxic and hyperoxic trials ( $P<0.05$ ; figure 6).  $T_{lim}$  was not different between BR and PL in the hyperoxic trials ( $P>0.05$ ,  $d=0.37$ ), tended to be longer with BR in the normoxic trials ( $P=0.087$ ,  $d=0.61$ ) and was increased with BR in the hypoxic trials ( $P<0.05$ ,  $d=1.13$ ; figure 6). The improved exercise tolerance after BR supplementation in hypoxia was negatively correlated with the end-exercise quadriceps TOI in the PL-Hypo trial ( $r = -0.78$ ,  $P<0.05$ ; figure 7).

## 4. DISCUSSION

In the present study, the effects of acute BR supplementation on quadriceps TOI,  $\dot{V}O_2$  kinetics and exercise tolerance were assessed in participants breathing normoxic, hyperoxic and hypoxic gas mixtures. The principal original findings of the present study were that BR supplementation improved severe-intensity exercise tolerance in hypoxia with a large effect size ( $d=1.13$ ), but did not improve severe-intensity exercise tolerance in normoxia ( $d=0.61$ ) or hyperoxia ( $d=0.37$ ). The increase in  $\dot{V}O_2$  above baseline at 180 s, as well as the peak  $\dot{V}O_2$  attained at  $T_{lim}$ , were greater in the severe-intensity step test completed in hypoxia, but not in normoxia or hyperoxia, following BR supplementation. The improvement in exercise tolerance following BR compared to PL supplementation in the severe-intensity step test completed in hypoxia was negatively correlated with quadriceps muscle oxygenation quadriceps TOI at  $T_{lim}$  in the PL condition. Collectively, these findings indicate that the degree to which BR supplementation improves severe-intensity exercise tolerance is influenced by the magnitude of skeletal muscle deoxygenation incurred. These original observations have important implications for improving understanding of the settings in which BR supplementation can be applied to enhance oxidative metabolism and exercise capacity.

Acutely ingesting 210 mL of BR, which provided a  $NO_3^-$  dose of 18.6 mmol, increased plasma  $[NO_2^-]$  in the present study consistent with numerous previous studies [11-15,18-20,24-28]. This circulating plasma  $NO_2^-$  can then impact physiological processes either through direct  $NO_2^-$  action [40,41] or through its subsequent reduction to NO via numerous ubiquitously expressed  $NO_2^-$  reductases [42]. While plasma  $[NO_2^-]$  declined during the exhaustive severe-intensity exercise test BR-Norm, BR-Hypo and BR-Hyper conditions, neither the plasma  $[NO_2^-]$  at exhaustion nor the absolute decline in plasma  $[NO_2^-]$  were different between these conditions. It has previously been reported that plasma  $[NO_2^-]$  declines during a similar extent during severe-intensity exercise in normoxia and normobaric hypoxia [Kelly et al., 2014, Am J Physiol Regul Integr Comp Physiol], consistent with the observations in the current study. Although previous studies [30,31] have reported a lowering in plasma [nitrite] in hyperoxia at rest, the comparable decline in plasma  $[NO_2^-]$  in the BR-Hyper condition relative to the BR-Norm and BR-Hypo conditions during severe-intensity exercise is an original contribution of this study. In addition to increasing plasma  $[NO_2^-]$  via BR supplementation, quadriceps muscle oxygenation was modulated in parallel with the different  $FIO_2$  conditions administered in the

current study, as confirmed using multi-channel NIRS. Specifically, compared to normoxia, TOI was increased in hyperoxia and lowered in hypoxia.

Although group mean  $\dot{V}O_2$  at the end of moderate-intensity exercise was lowered after BR supplementation by 3%, 2% and 1% in hypoxia, normoxia and hyperoxia compared to the respective PL trials, these differences did not attain statistical significance. The lack of an improvement in submaximal exercise economy after BR supplementation in hyperoxia is an original finding, but the lack of an improvement in submaximal exercise economy after acute BR supplementation in normoxia and hypoxia conflicts with our experimental hypothesis and with some [19,20,27,43,44], but not all [45], previous observations in moderately fit individuals. This lack of an effect of acute BR supplementation in normoxia and hypoxia could be the result of completing a single moderate-intensity step test instead of averaging the  $\dot{V}O_2$  responses to two moderate-intensity step tests to improve the signal-to-noise ratio in the  $\dot{V}O_2$  response [19,20,24].

It is known that NIRS-derived oxygenation variables exhibit significant spatial heterogeneity in the quadriceps muscles [46]. Most previous studies have used single channel NIRS to assess the effect of BR supplementation on tissue oxygenation [12,24,27,44]. Therefore, an important original contribution of the current study is the utilisation of three NIRS channels on the quadriceps to provide a more complete profile of quadriceps TOI and how this might be modulated by BR supplementation when inhaling hypoxic, normoxic and hyperoxic air. Consistent with our experimental hypothesis, quadriceps TOI during the U→M step exercise test was increased after BR supplementation compared to PL in normoxia, but not hyperoxia. However, quadriceps TOI during the U→M step exercise test was not different between the BR and PL conditions in hypoxia, which conflicts with our experimental hypothesis and previous reports of improved lower limb muscle oxygenation after BR supplementation [24,26,44]. Nevertheless, and in accord with the observations of the current study, greater improvements in cerebral oxygenation have been observed in normoxia compared to hypoxia [47], and there is some evidence that the vasodilation conferred by an increase in circulating  $NO_2^-$  is greatest in normoxia and blunted in hypoxia and hyperoxia [48]. Therefore, our data do not support the notion that acute BR supplementation is more effective at improving cycling economy and quadriceps TOI during moderate-intensity exercise in hypoxia compared to normoxia, but suggest that the improvements in quadriceps TOI during moderate-intensity

exercise in normoxia following BR supplementation are blunted in both hypoxia and hyperoxia.

During the M→S step test, the change in  $\dot{V}O_2$  from the moderate-intensity baseline to 180 s of severe-intensity cycling was not different in normoxia or hyperoxia, but was increased by 8% in hypoxia, following BR compared to PL supplementation. Similarly, the peak  $\dot{V}O_2$  attained at  $T_{lim}$  in the M→S step test was increased in the BR condition compared to PL by 5% in hypoxia but was not different in normoxia or hyperoxia. The lack of an effect of BR supplementation on  $\dot{V}O_2$  kinetics during severe-intensity exercise in normoxia is consistent with some previous reports [20]. However, consistent with the observations of the current study, it has recently been reported that chronic BR supplementation can increase mean  $\dot{V}O_2$  and the fractional utilisation of  $\dot{V}O_{2max}$  in trained cyclists completing a 10 km cycling time trial in hypoxia [49]. The increase in  $\dot{V}O_2$  from the moderate-intensity baseline to 180 s of severe-intensity cycling and at the end of severe-intensity cycling in hypoxia following BR supplementation was not accompanied by altered quadriceps TOI over the corresponding time frames. Since TOI provides insight into the matching between tissue  $O_2$  supply and utilisation [50], these data imply that a proportional increase in skeletal muscle  $O_2$  supply permitted the increase in  $\dot{V}O_2$ .

The tolerable duration of severe-intensity exercise after BR supplementation was not significantly improved (+ 4%) in hyperoxia or normoxia (+ 6%), but was enhanced with a large effect size in hypoxia (+ 8%), compared to the respective PL trials. A greater improvement in severe exercise tolerance after BR supplementation in hypoxia compared to normoxia has been reported previously, but without changes in  $\dot{V}O_2$  between BR and PL [24]. Conversely, our findings conflict with studies reporting that  $NO_3^-$  supplementation is ineffective at enhancing performance in normoxia and hypoxia [Bourdillon et al. 2015, Front Physiol; MacLeod et al., 2015, Int J Sport Nutr Exerc Metab; Nybäck et al., 2017, Nitric Oxide] and equally effective at enhancing performance in normoxia and hypoxia [Rokkedal-Lausch et al., 2019, Nitric Oxide]. Instead, BR supplementation improved severe-intensity exercise tolerance in the current study concomitant with an increase in  $\dot{V}O_2$  during the initial stages of exercise as well as the attainment of a greater  $\dot{V}O_{2peak}$  at  $T_{lim}$  in hypoxia. Therefore, a greater oxidative energy contribution is likely to have contributed to the improved severe-intensity exercise tolerance in hypoxia after BR supplementation in the current study. This increase in oxidative energy turnover would be expected to blunt the decline in finite anaerobic energy reserves and the

accumulation of metabolites implicated in the process of fatigue permitting a greater tolerable duration of exercise [51]. Indeed, it has been reported that quadriceps [PCr] and pH decline, and [ADP] and [Pi] increase, to a lesser extent following BR supplementation in hypoxia concomitant with an increase in exercise tolerance [25]. However, we cannot exclude the possibility that BR supplementation might have enhanced severe exercise tolerance and permitted the attainment of a higher  $\dot{V}O_{2\text{peak}}$  in hypoxia by attenuating peripheral fatigue development via improved myofiber  $\text{Ca}^{2+}$  handling [35] or modulating aspects of central fatigue, which is known to make a greater contribution to fatigue in hypoxia [52]. It should also be acknowledged that the lack of a statistically significant improvement in  $T_{\text{lim}}$  following acute BR supplementation in the current study might be linked insufficient statistical power.

The extent to which severe-intensity exercise tolerance was improved following BR supplementation in hypoxia was negatively correlated with quadriceps TOI at  $T_{\text{lim}}$  in the PL-Hypo condition. Combined with the observations that severe-intensity exercise tolerance was only improved in hypoxia, these findings indicate that the ergogenic potential of BR supplementation during severe-intensity exercise is linked to the degree of skeletal muscle deoxygenation incurred during such exercise. These observations have implications for improving understanding of the exercise settings in which BR supplementation is more likely to be effective at improving exercise capacity. Specifically, it is possible that BR supplementation is more effective at improving performance at altitude, in individuals with a greater proportion of type II (fast-twitch) skeletal muscle [53,54] and in patients with disorders of skeletal muscle  $\text{O}_2$  delivery [54,55]. Moreover, since  $\dot{V}O_{2\text{peak}}$  is an important predictor of all-cause mortality [56] and is attenuated in numerous disease conditions that engender hypoxia [e.g., 57], an increase in  $\dot{V}O_{2\text{peak}}$  in hypoxia after acute BR supplementation might have implications for improving exercise capacity, quality of life and mortality in certain patient groups. However, it should be acknowledged that, while well-trained endurance athletes exhibit increased muscle  $\text{O}_2$  extraction during exercise in hypoxia leading to greater muscle deoxygenation (Van Thienen and Hespel, 2016, J Appl Physiol), there is some evidence that  $\text{NO}_3^-$  supplementation does not enhance endurance performance in well-trained athletes in hypoxia (Bourdillon et al. 2015, Front Physiol; MacLeod et al., 2015, Int J Sport Nutr Exerc Metab; Nybäck et al., 2017, Nitric Oxide). Therefore, further research is required to evaluate the  $\text{PO}_2$ -dependency of the potential beneficial effects of  $\text{NO}_3^-$  supplementation on physiological responses and performance during exercise in different populations.



In conclusion, acute ingestion of BR, which increased circulating plasma  $[\text{NO}_2^-]$ , did not significantly improve moderate-intensity cycling economy in normoxia, hypoxia or hyperoxia, but increased quadriceps TOI in the former. During severe-intensity cycling, BR ingestion improved exercise tolerance in hypoxia, but did not improve exercise tolerance in normoxia or hyperoxia. The improvement in severe-intensity exercise tolerance with BR supplementation in hypoxia was negatively correlated with quadriceps TOI such that those who experienced greater quadriceps deoxygenation in hypoxia exhibited the greatest improvement in exercise tolerance with BR ingestion. These original findings indicate that the ergogenic potential of acute BR supplementation is increased as the skeletal muscle becomes increasingly deoxygenated.

### **Acknowledgement**

The authors are grateful to Jamie Blackwell, Thomas Beadle, Felix Coates and Louis Bouwers for their assistance with data collection and analysis.

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## Figure Legends

**Figure 1:** Plasma nitrite concentration ( $[\text{NO}_2^-]$ ) following acute ingestion of nitrate-depleted beetroot juice (PL) in hypoxia (PL-Hypo), normoxia (PL-Norm) and hyperoxia (PL-Hyper), and following acute ingestion of nitrate-rich beetroot juice (BR) in hypoxia (BR-Hypo), normoxia (BR-Norm) and hyperoxia (BR-Hyper). Plasma was sampled at the end of unloaded baseline cycling at 20 W, moderate-intensity cycling, 120 s of severe-intensity cycling and at limit of tolerance ( $T_{\text{lim}}$ ) during severe-intensity cycling. Data are presented as group mean  $\pm$  SEM. \* indicates BR-Hypo, BR-Norm, and BR-Hyper trials are higher than PL-Hypo and PL-Norm and PL-Hyper ( $P < 0.05$ ). # indicates different from baseline, end moderate and 120 s severe in the BR-Hypo, BR-Norm, and BR-Hyper trials ( $P < 0.05$ ).

**Figure 2:** Arterial oxygen saturation ( $\text{SpO}_2$ ) following acute ingestion of nitrate-depleted beetroot juice (PL) in hypoxia (PL-Hypo), normoxia (PL-Norm) and hyperoxia (PL-Hyper), and following acute ingestion of nitrate-rich beetroot juice (BR) in hypoxia (BR-Hypo),



normoxia (BR-Norm) and hyperoxia (BR-Hyper). The open bars represent the group mean  $\pm$  SEM SpO<sub>2</sub> from the PL trials while the filled bars represent group mean  $\pm$  SEM SpO<sub>2</sub> from the BR trials. Data represent the mean SpO<sub>2</sub> up to 120 s of severe-intensity cycling exercise. \* indicates different from PL-Hypo and BR-Hypo ( $P<0.05$ ).

**Figure 3:** Quadriceps tissue oxygenation index (TOI) responses following acute ingestion of nitrate-depleted beetroot juice (PL) in hypoxia (PL-Hypo), normoxia (PL-Norm) and hyperoxia (PL-Hyper). TOI data during unloaded baseline cycling at 20 W (-60-0 s), moderate-intensity cycling (0-240 s) and severe-intensity cycling (240-360 s) are group mean responses displayed as 5 s averages. TOI data at limit of tolerance ( $T_{lim}$ ) during severe-intensity cycling exercise represent the mean TOI over the final 60 s of exercise and are presented as group mean  $\pm$  SEM. # indicates different from PL-Hypo and BR-Hypo ( $P<0.05$ ). \* indicates different from PL-Norm, BR-Norm, PL-Hypo and BR-Hypo ( $P<0.05$ ).

**Figure 4:** Quadriceps tissue oxygenation index (TOI) responses following acute ingestion of nitrate-depleted beetroot juice (PL) and nitrate-rich beetroot juice (BR) in hypoxia (upper panel; PL-Hypo and BR-Hypo, respectively), normoxia (middle panel; PL-Norm and BR-Norm, respectively) and hyperoxia (lower panel; PL-Hyper and BR-Hyper, respectively). TOI data during unloaded baseline cycling at 20 W (-60-0 s), moderate-intensity cycling (0-240 s) and severe-intensity cycling (240-360 s) are group mean responses displayed as 5 s averages. TOI data at limit of tolerance ( $T_{lim}$ ) during severe-intensity cycling exercise represent the mean TOI over the final 60 s of exercise and are presented as group mean  $\pm$  SEM. \* indicates different from PL-Hypo ( $P<0.05$ ). # indicates different from PL-Norm ( $P<0.05$ ).

**Figure 5:** Pulmonary oxygen uptake ( $\dot{V}_{O_2}$ ) responses following acute ingestion of nitrate-depleted beetroot juice (PL) and nitrate-rich beetroot juice (BR) in hypoxia (upper panel; PL-Hypo and BR-Hypo, respectively), normoxia (middle panel; PL-Norm and BR-Norm, respectively) and hyperoxia (lower panel; PL-Hyper and BR-Hyper, respectively).  $\dot{V}_{O_2}$  data during moderate-intensity cycling (-60-0 s) and severe-intensity cycling (240-360 s) are displayed as 5 s averages from a representative subject. Insets present end-exercise  $\dot{V}_{O_2}$  (mean over the final 60 s of severe-intensity cycling) following PL and BR supplementation in hypoxia, normoxia and hyperoxia. The open bars represent the group mean  $\pm$  SEM end-exercise  $\dot{V}_{O_2}$  in the PL trials, while the filled bars represent the group mean  $\pm$  SEM plasma end-exercise  $\dot{V}_{O_2}$  in the BR trials. The solid grey lines represent the individual changes in end-exercise  $\dot{V}_{O_2}$

following BR supplementation at a given fraction of inspired O<sub>2</sub>. \* indicates different from PL-Hypo ( $P<0.05$ ).

**Figure 6:** Time to limit of tolerance ( $T_{lim}$ ) following acute ingestion of nitrate-depleted beetroot juice (PL) in hypoxia (PL-Hypo), normoxia (PL-Norm) and hyperoxia (PL-Hyper), and following acute ingestion of nitrate-rich beetroot juice (BR) in hypoxia (BR-Hypo), normoxia (BR-Norm) and hyperoxia (BR-Hyper). The open bars represent the group mean  $\pm$  SEM  $T_{lim}$  from the PL trials while the filled bars represent the group mean  $\pm$  SEM  $T_{lim}$  from the BR trials. The solid grey lines represent the individual changes in  $T_{lim}$  following BR supplementation at a given fraction of inspired O<sub>2</sub>. \* indicates different from PL-Hypo ( $P<0.05$ ). # indicates different from PL-Hypo and BR-Hypo ( $P<0.05$ ). ¥ indicates different from PL-Norm, BR-Norm, PL-Hypo and BR-Hypo ( $P<0.05$ ).

**Figure 7:** The relationship between end-exercise tissue oxygenation index (TOI; mean over the final 60 s of severe-intensity cycling) following nitrate-depleted beetroot juice (PL) in hypoxia (PL-Hypo) and the change ( $\Delta$ ) in time to limit of tolerance ( $T_{lim}$ ) between the PL and nitrate-rich beetroot juice (BR) trials in hypoxia (PL-Hypo and BR-Hypo, respectively). Note that end-exercise TOI in PL-Hypo was negatively correlated with  $\Delta T_{lim}$  between the PL-Hypo and BR-Hypo trials.

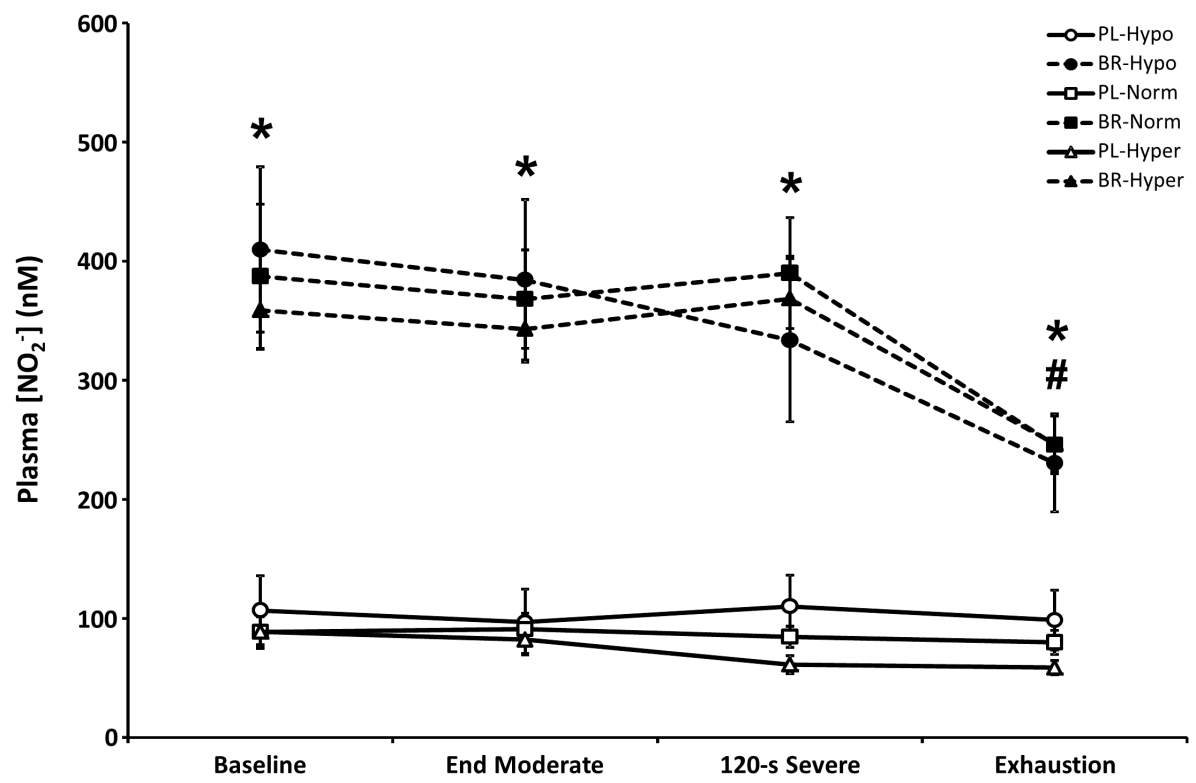


Fig. 1

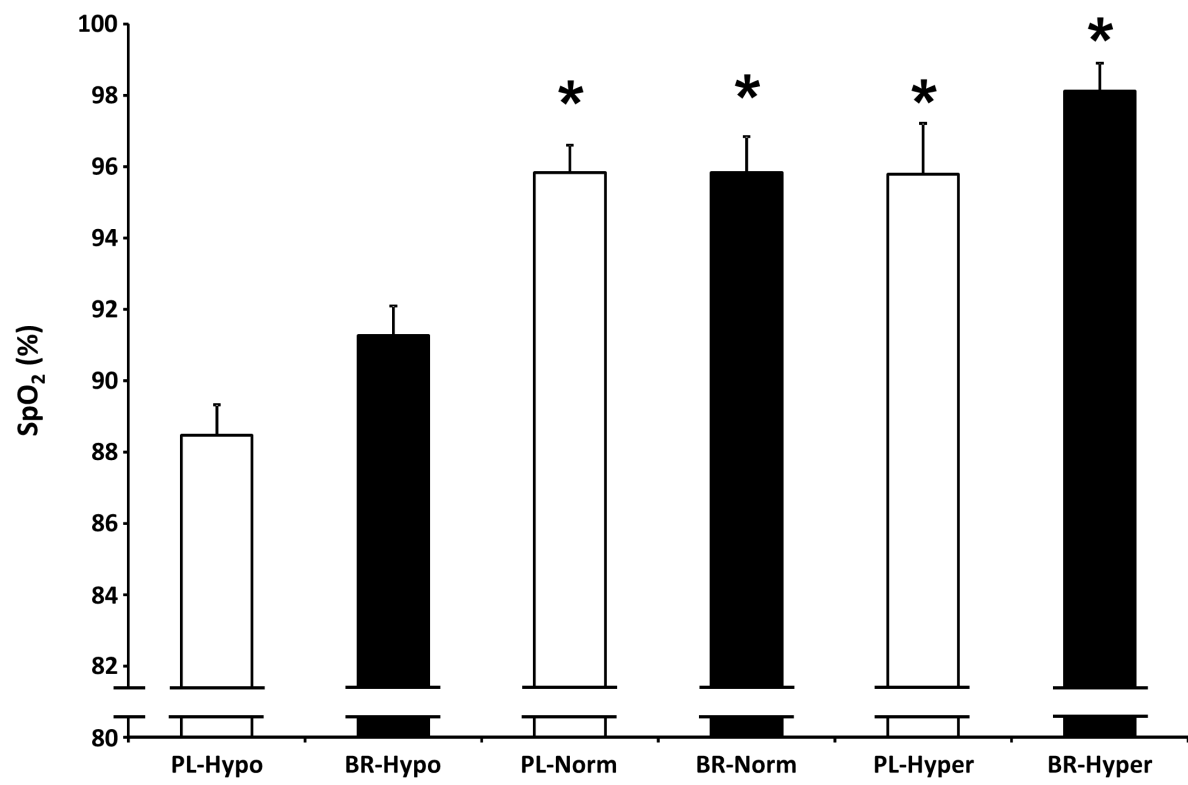


Fig. 2

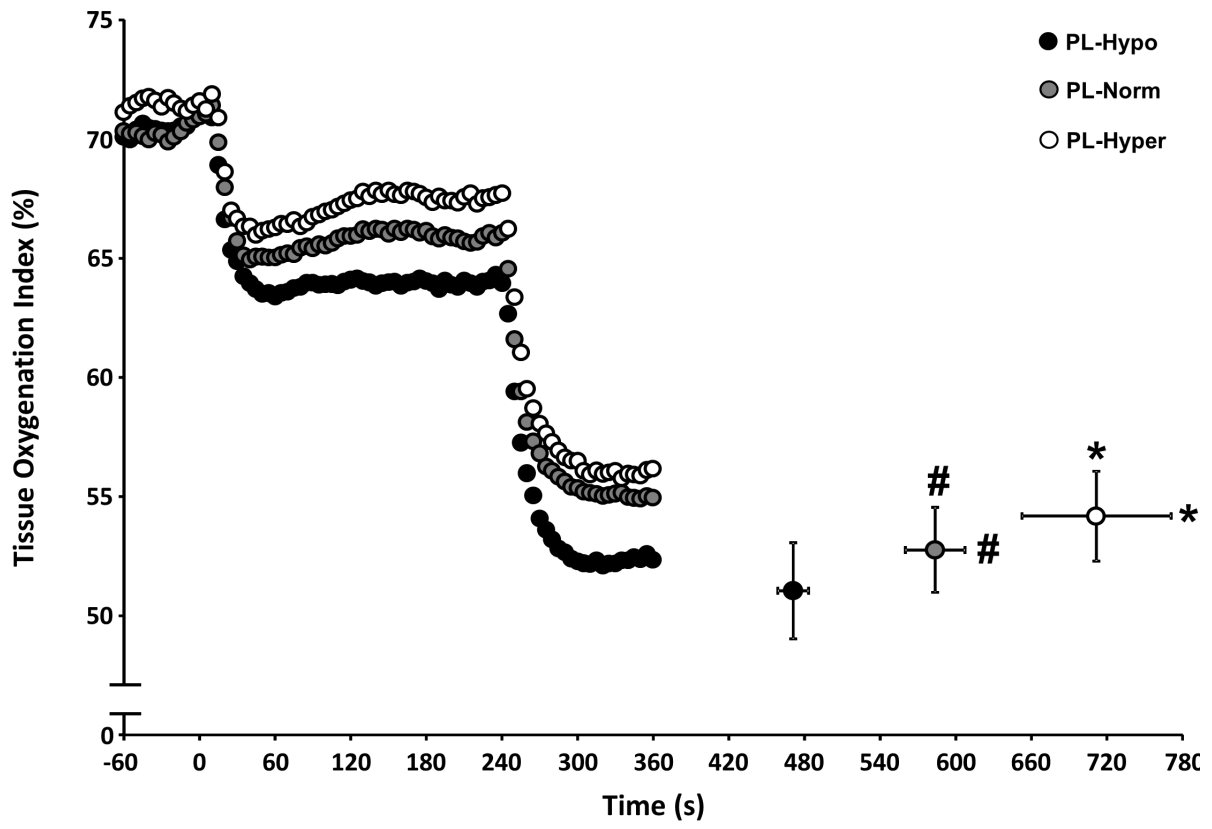


Fig. 3

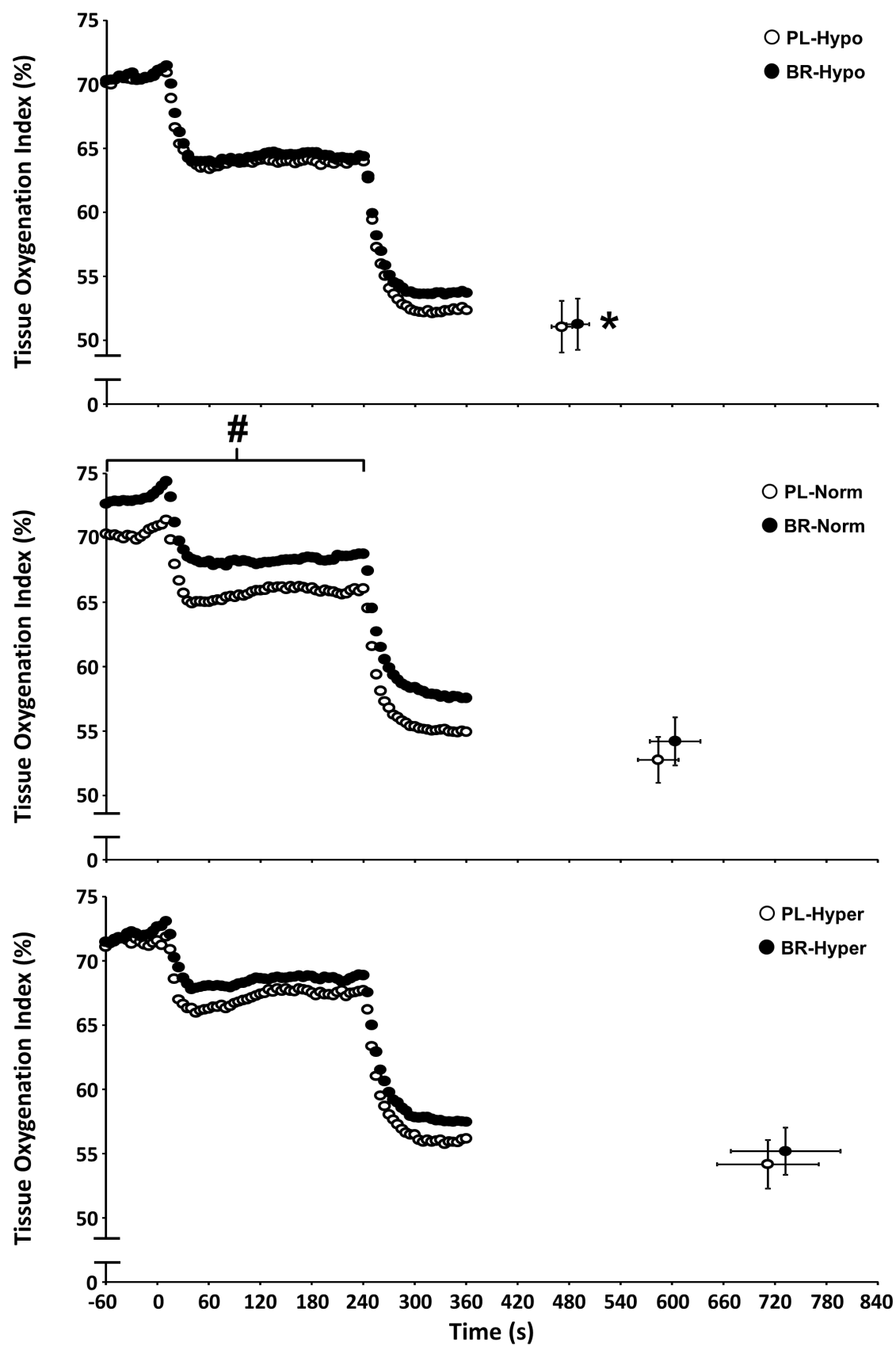


Fig. 4

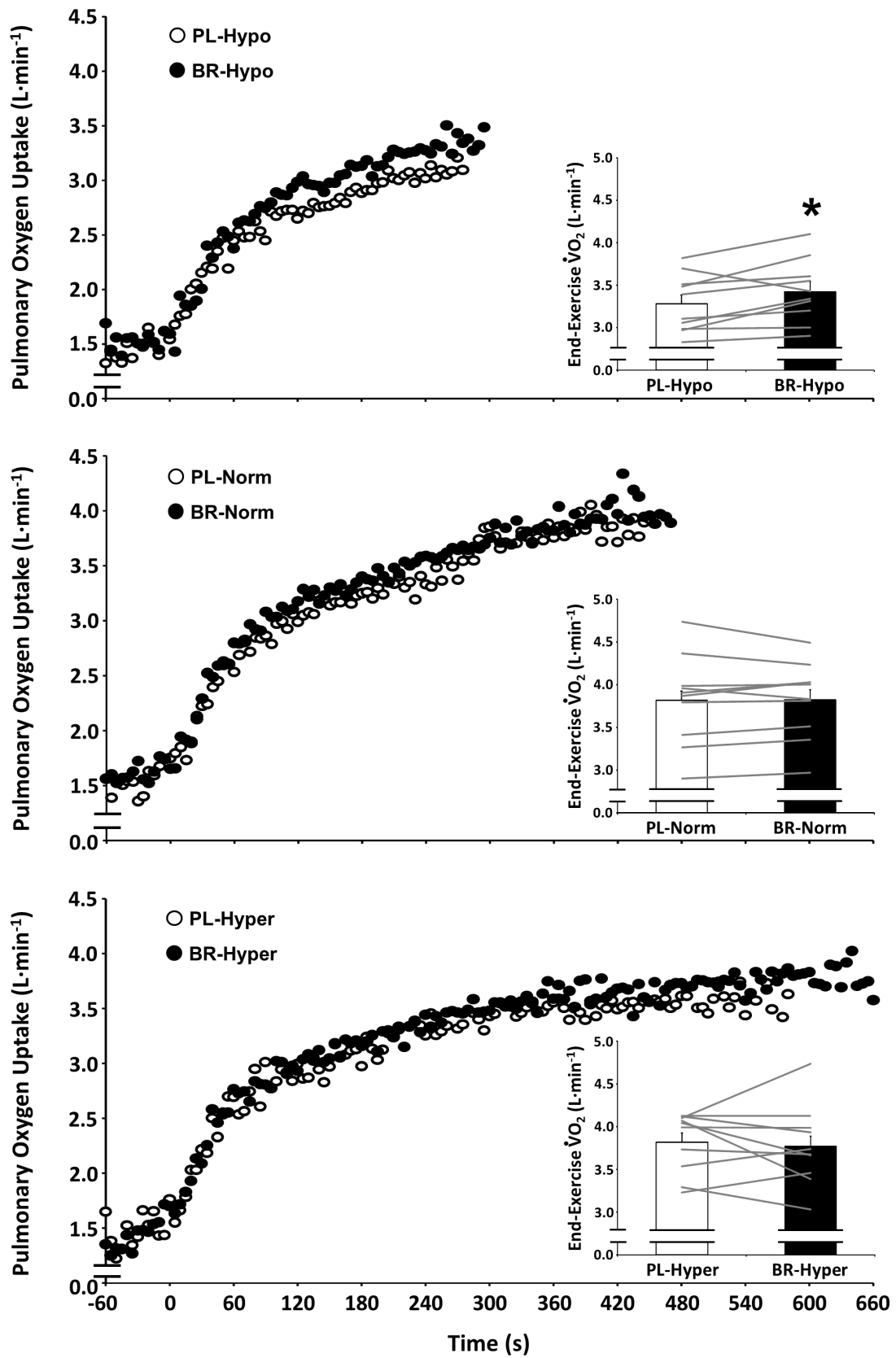


Fig. 5

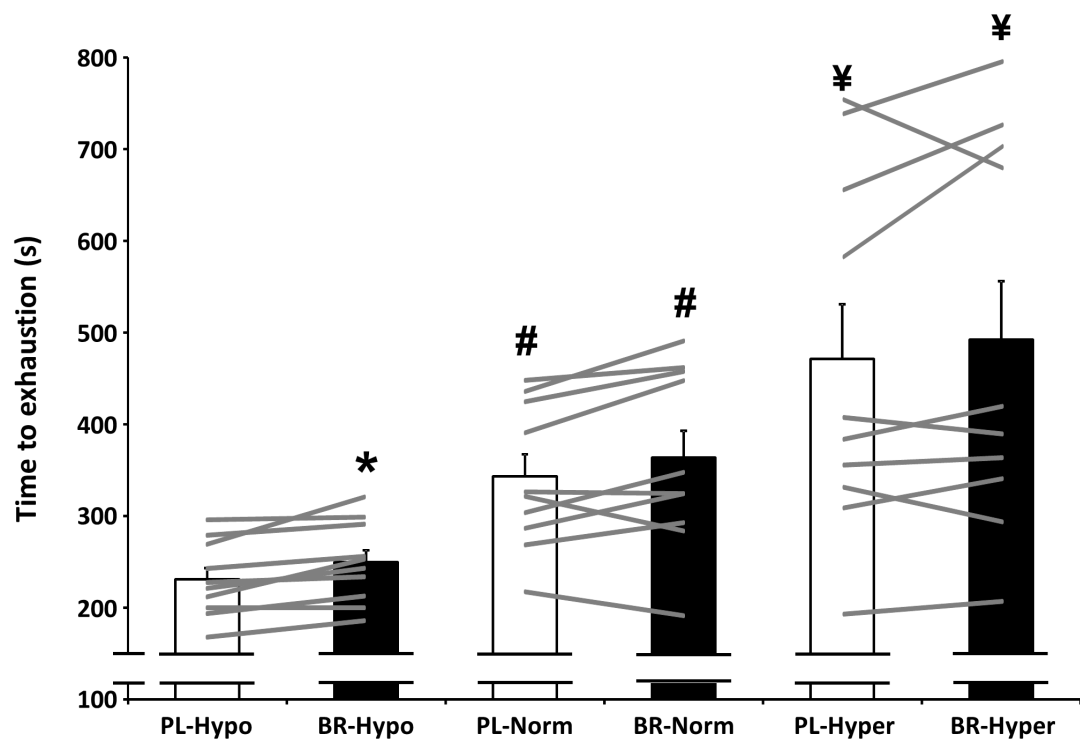


Fig. 6



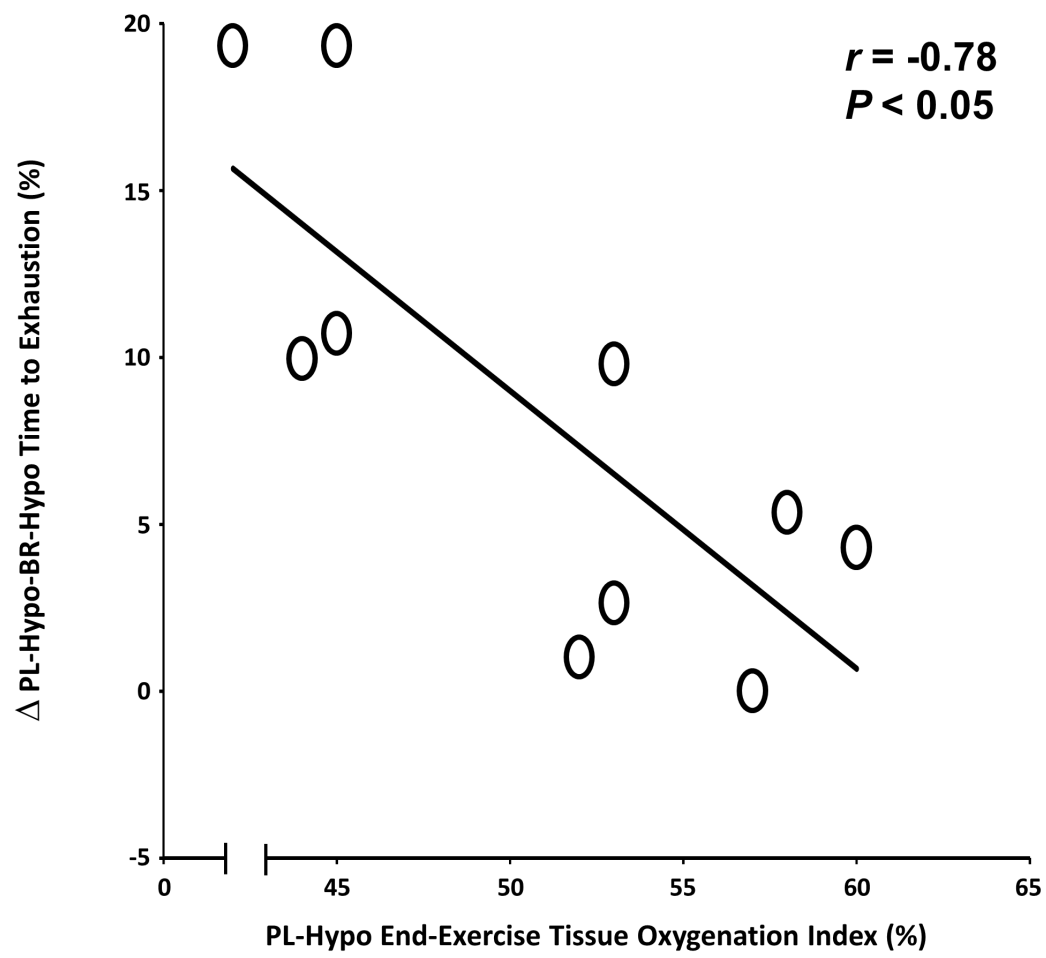


Fig. 7